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Concentration of immunoreactive progesterone and androgens in the yolk of hens' eggs *(Gallus domesticus)*

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Summary

The theca and granulosa cells of the follicular wall produce gonadal steroids. We investigated the progesterone and androgene concentrations in the yolk layers to gain information on the concentrations of these hormones, as chicken embryos are exposed to this environment.

After extraction, the mean concentrations of immunoreactive progesterone and androgens in the yolk of twenty eggs were measured. In another six yolks, cross-section disks from the whole frozen yolks (2 mm thick) were prepared, six concentric layers separated and the values of progesterone, androstenedione and testosterone determined using enzyme immunoassays (EIAs). The extracts were separated by straight phase HPLC and the immunoreactive substances were determined in all fractions to validate the assays. Using the progesterone EIA, three immunoreactive substances were found. Immunoreactive androstenedione was the dominating androgen.The mean concentrations of immunoreactive progesterone were 13.7 ± 2.9 µg/yolk and of androstenedione 1.2 ± 0.7 µg/yolk, respectively. The yolk contained highest progesterone concentrations in the outer layer (693 ± 55 ng/g). The concentrations decreased reaching 14.1 ± 8.7 ng/g yolk in the centre. The lowest androstenedione concentrations were measured in the surface layer (61.3 ± 25.1 ng/g yolk). The values increased reaching a maximum in layer 4 (131.3 ± 35.7 ng/g) and decreased in the following two layers. We conclude that the steroid concentrations in different layers of the yolk represent the metabolic capacity of theca and granulosa cells during the stages of follicular maturation.

Schlüsselwörter: Eier, Vögel, Progesteron, Androgene.

Zusammenfassung

Konzentration von immunreaktivem Progesteron und Androstendion im Dotter von Hühnereiern

Die Theka- und Granulosazellen des Follikels bilden Sexualsteroide. Ziel unserer Untersuchungen war es, die Konzentrationen von Progesteron und Androgenen in Hühnereiern zu messen.

In Dotterproben von 20 Hühnereiern wurde die durchschnittliche Konzentration von immunreaktivem Progesteron und Androgenen gemessen. Bei weiteren 6 Eiern wurde aus der gefrorenen Dotterkugel eine 2 mm dicke Schicht herausgeschnitten, 6 konzentrische Schichten abgehoben und die Konzentrationen von Progesteron, Androstendion und Testosteron mit Enzymimmunoassays (EIA) gemessen. Zur Validierung der Tests wurden einzelne Proben nach Extraktion mittels Hochdruckflüssigkeitschromatographie getrennt und die immunreaktiven Substanzen in den einzelnen Fraktionen gemessen.

Mit dem Progesteron-EIA wurden 3 immunreaktive Substanzen nachgewiesen. Androstendion war das mengenmäßig gegenüber Testosteron überwiegende Androgen, und es eluierte chromatographisch als ein Peak.

Die mittlere Progesteronkonzentration im Dotter betrug 13,7± 2,9 µg/ Dotter, die Androstendionkonzentration 1,2 ± 0,7 µg. Die Außenschicht enthielt die höchste Progesteronkonzentration (693 ± 55 ng/g). Die Konzentration nahm zur Mitte hin ab und betrug im Zentrum 14,1 ± 8,7 ng/g. Hingegen wurde die niedrigste Androstendionkonzentration in der Außenschicht gemessen (61,3 ± 25,1 ng/g Dotter). Die Konzentrationen stiegen dann an, erreichten in Schicht 4 das Maximum (131,3 ± 35,7 ng/g) und sanken zur Mitte hin wieder ab. Es ist daher anzunehmen, daß die Steroidkonzentrationen in den jeweiligen Schichten die metabolische Aktivität der Theka- und Granulosazellen widerspiegeln.

Introduction

The ovaries of egg-laying hens contain a hierarchy of developing yolk-filled follicles. Approximately once per day one follicle ruptures and the ovum including the yolk is released. ETCHES et al. (1983) found that the largest follicle of the hierarchy acquired responsiveness to gonadotropin releasing hormone (GnRH) between 10 and 16 hours after the previous ovulation. The authors also demonstrated the overall growth of the follicle up to the period within one hour of ovulation.

The egg yolk consists of yellow and white yolk. The white yolk originates from white (non-mature) follicles. Yellow yolk consists of two types of lipoprotein emulsions, the light yellow yolk and the deep yellow yolk (OKUBO et al., 1997). During follicular maturation, the white follicle accumulates yellow yolk (7 - 12 days before ovulation) and is then described as yellow follicle. A rapid increase in yellow yolk follows till one day before ovulation (OKUBO et al., 1997).

The transfer of gonadal steroids between the periphe-

ral circulation and the yolk seems to be limited. ARCOS (1972) injected 40 µCi of 17β oestradiol or progesterone in one single intravenous injection. Eggs were collected daily for ten days and the radioactivity of each yolk was measured. A steady value was reached (0.04 % of the injected dose) by four to seven days.

HUANG and NALBANDOV (1979) incubated theca and granulosa cells of chicken and found that the granulosa cells produced progesterone and testosterone. The role of theca cells in steroidogenesis remained unclear.

Progesterone, the major steroid product of granulosa cells in hens is produced from pregnenolone and the 3β-hydroxysteroid dehydrogenase activity increased in individual cells as follicles matured (MARRONE and SEBRING, 1989). Progesterone can be converted into androgens by side chain cleavage. In some bird species, as for example the canary (*Serinus canaria*), the testosterone concentrations in yolk increased in the later formed eggs of a clutch (SCHWABL, 1996). The concentration of gonadal steroids in yolk is described to vary in some species, as for example in the dark-eyed junco and in the red-winged blackbird (LIPAR et al., 1999). The authors found higher progesterone concentrations in exterior layers of the yolk, whereas intermediate layers contained highest testosterone concentrations.

We measured progesterone and androgens in the yolk of hens' eggs to gain information on the hormone concentration as eggs are an important food for human consumption.

Another reason for this study was that maternal androgens were reported to be deposited in the yolk in proportion to systemic levels (SCHWABL, 1996). As circulating steroids are known to be modulated by environmental or social stimuli and, on the other hand, yolk androgens seem to affect personality/coping styles of the offspring, it is relevant to know the variability of steroids and their distribution among yolks. The question is, whether systemic steroids enter the yolk directly, via passive diffusion, or whether they may affect steroid synthesis in the theca/granulosa cells. In the first case, due to the interactive dynamics of systemic steroid fluctuations and yolk synthesis, one would predict a considerable variability in the distribution of steroids among yolks. In the case of intervening synthesis by the theca/granulosa cells, the steroid distribution patterns in different yolks should be rather regular.

Material and Methods

Eggs and yolk extraction

To quantify the total concentrations of progesterone and androgens in yolk, twenty fresh eggs were frozen until analysis. After thawing, weighing and mixing of individual yolks, 0.5 g of each yolk was suspended in 1.5 g of water and vortexed for 30 s. Afterwards, the suspension was diluted with 8 ml methanol and vortexed for 30 min. The sample was centrifuged at -10 °C and 1 ml of the supernatant was transfered into a new vial and diluted with 9 ml of water. The sample was then extracted using Sep-Pak® C_{18} cartridges (Fa. Waters, Milford, USA). After passing the sample through the primed cartridge, the mini-column was washed with 10 ml of water, and the steroids were eluted from the cartridge with 5 ml of 80 % methanol. The extract was evaporated and re-dissolved in 1 ml of assay buffer.

Preparation of yolk layers

In another six yolks the distribution of steroid hormones within the yolk was investigated. A cross-section disk from the whole frozen yolk (2 mm thick) was prepared for the dissection of the yolk layers. The part of the yolk, where the dissection was begun, was deformed (not circular) and therefore this half of the yolk was not used for analysis. By using a pair of compasses concentric lines were carved into the other half of the yolk disk and the concentric layers were transfered into vials, starting with the outer layers. The clean-up procedure was the same as described above, but smaller amounts of yolk were used for the assays.

Enzyme immunoassays for progesterone, androstenedione and testosterone were used to measure the hormone concentrations. The validity of the tests was checked by high performance liquid chromatography (HPLC) separation of the samples (after Sep-Pak® extraction).

The extract was dissolved in chloroform/n-hexane (70/30) and injected onto a column (Seibersdorf® Si 60 [Forschungszentrum Seibersdorf, Austria]). The chromatography started with chloroform/n-hexane (70/30) for 25 min. Afterwards, a methanol gradient was used (linear in 5 min up to 6 % methanol, in the next 5 min up to 10 % methanol) to elute more polar substances.

Enzyme immunoassays

Enzyme immunoassays for progesterone and other 20-oxopregnanes (SCHWARZENBERGER et al., 1996) and androgens (PALME and MÖSTL, 1993) were used. In the case of the assay for 17-oxoandrogens, androstenedione was used as standard instead of epiandrosterone, as this steroid was the dominating androgen in yolk (see results).

Results

After HPLC separation and measurement of the individual fractions using the progesterone-EIA, three major peaks were seen (Fig.1). The immunoreactive substance causing the third peak showed the same chromatographic mobility as progesterone. The androstenedione EIA reacted with one substance with an elution pattern like androstenedione (data not shown). By measuring the total yolk concentrations, mean values of 13.7 ± 2.9 µg progesterone per yolk and 1.2 ± 0.7 µg androstenedione were found. There was a positive correlation ($p < 0.001$, Fig. 2) between the progesterone concentration expressed as ng/g and as ng/ yolk ($r\approx = 0.66$), and an even closer positive correlation in androstenedione ($p < 0.001$, $r\approx = 0.98$).

The six layers into which we dissected yolk disks contained highest progesterone concentrations (693 ± 55 ng/g) in the outer layers (Fig. 3). The concentrations decreased toward the centre. The dominating androgen measured was androstenedione, the testosterone concentrations were much lower (Fig. 4). Lowest androstenedione concentrations were measured in the surface layer (61.3 ± 25.1 ng/g yolk). The values increased reaching a maximum in layer 4 (131.3 ± 35.7 ng/g), and in the following layers the values decreased (Fig 4). Using the Kruskal-Wallis test, differences in the median values among the hormone concentrations in the different layers were found to be greater than would be expected by chance (androstenedione $p = 0.002$, testosterone $p = 0.004$).

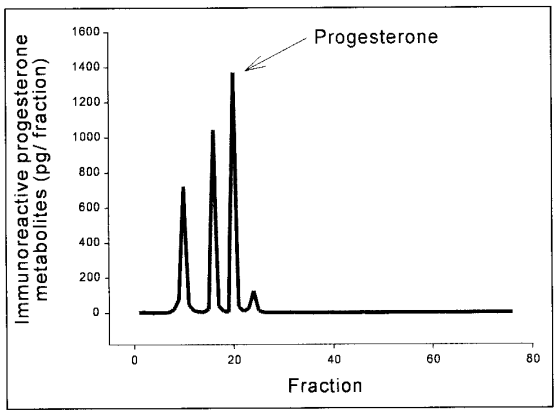
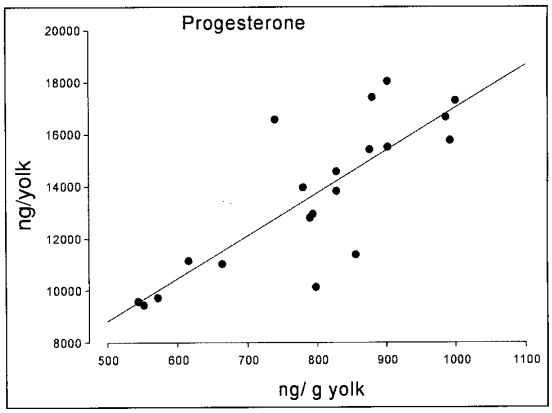


Fig. 1: Straight phase high performance liquid chromatography of a yolk extract; the arrow indicates the fraction in which the progesterone standard eluted from the column.



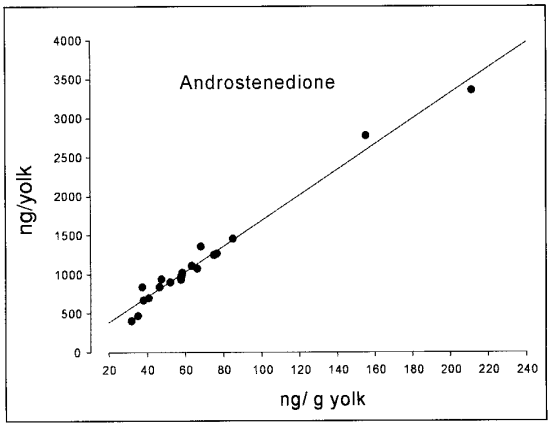


Fig. 2: Correlation between the progesterone and androstenedione concentration in yolk expressed as ng/g (x-axis) and ng / yolk (y-axis)

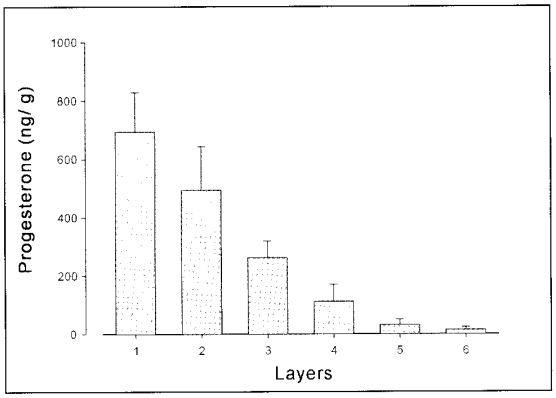
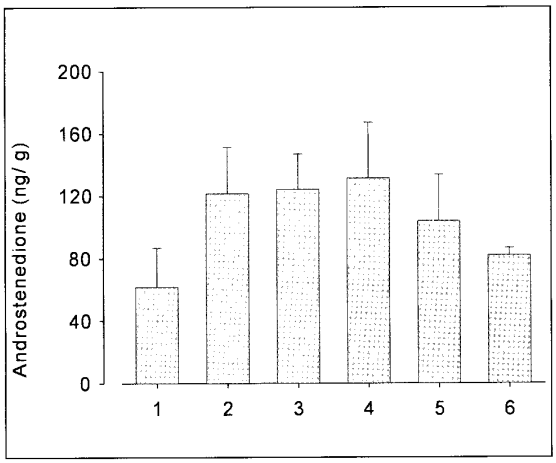


Fig. 3: Progesterone concentrations ($\bar{x} \pm s_{\bar{x}}$) in the layers of six yolks; layer 1 represents the part of the yolk closest to the surface, layer 6 the centre of the yolk disk.



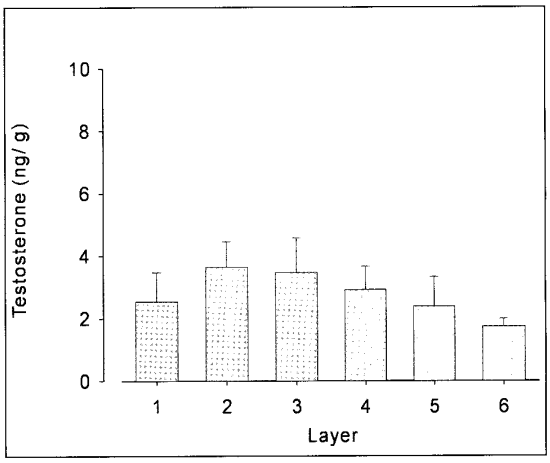


Fig. 4: Androstenedione (left graph) and testosterone (right graph) concentrations ($\bar{x} \pm s_{\bar{x}}$) in the layers of the yolk (n = 6)

Discussion

As seen in the HPLC immunograms, three immunoreactive progesterone metabolites were present. As the third peak had the same elution pattern as progesterone, it is likely that this peak represents progesterone. The other more non-polar substances are probably 5α-and 5β-pregnane,3,20-dione. Androgens were also present in yolk in substantial amounts. Androstenedione and testosterone can both be used as substrate for the aromatising enzymes

to form oestrogens. The dominating androgen androstenedione has much less androgenic activity than testosterone, but the conversion requires only a single enzymatic step and can be caused, for example, by the developing embryo.

The source of the gonadal steroids in yolk are theca or granulosa cells (for review see WITTMANN, 1981). As in mammals, the follicle contains high concentrations of gonadal steroids. There is a close correlation between progesterone and androstenedione concentrations in yolk expressed as ng/g and ng per yolk. This finding validates the method of taking yolk samples via biopsy but care has to be taken to sample the same layer.

The increasing progesterone concentrations from the centre to the outer layers represent the increasing progesterone production of the cells of the follicular wall. This may be accompanied by a decrease in 17 α hydroxylase or 17-20 lyase activity, similar to that described in the corpus luteum of mammals (GOWER and FOTHERBY, 1975). The distribution pattern of gonadal steroids in the various layers of yolk was similar in the dark-eyed junco and in the red-winged blackbird as described by LIPAR et al. (1999).

In hens, the concentrations of testosterone and progesterone in the peripheral plasma begin to rise between nine and six hours before ovulation (ETCHES and CHENG, 1981). In hens, progesterone and luteinizing hormone (LH) are interrelated in a positive feedback system (ETCHES and CUNNINGHAM, 1976). Like in mammals, the ovary produces steroid hormones.

ETCHES and DUKE (1984) described that in granulosa cells there is a peak of progesterone concentration four hours before ovulation, whereas the androstenedione content in theca and granulosa cells had a maximum about eight hours before ovulation. The authors concluded that the synthesis of androstenedione and oestradiol ceases in both follicular cell types after the follicle has been exposed to the preovulatory LH surge.

The regular distribution and the differences between progesterone and androgens of steroids found renders it unlikely that these enter the yolk in substantial amounts via passive diffusion from the maternal circulation. Rather, systemic hormones may affect the steroid synthesis or the transfer of theca/granulosa cells. Thus, environmental modulation of maternal systemic steroids probably does not directly affect the offsprings' personality/coping style development suggested by SCHWABL (1996), but only via an additional intervening synthesis.

In humans, the intake of gonadal steroids by eating eggs (13.7 µg/yolk) will most probably not interfere with any regulatory mechanisms, as the daily production of progesterone is 0.6 mg/day in men and women excrete 3.4 mg of androstenedione and about 0.35 mg of testosterone per day (GOWER, 1975).

An interesting aspect of the high progesterone concentration in yolk is the use of yolk for diluting semen. About 10-20 % of this dilution fluid consists of yolk. By using this dilution fluid, high amounts of progesterone and related substances influence semen cells. JAISWAL et al. (1999) showed that progesterone might be a week chemoattractant and may cause human sperm accumulation mainly by inducing hyperactivation - such as motility. By using different parts of yolk, the influence of progesterone and progesterone metabolites on sperm cells can be investigated.

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